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Oligoclonal Expansions of CD4⁺ and CD8⁺ T-Cells in the Target Organ of Patients With Biliary Atresia

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Abstract

Background & Aims: Biliary atresia is an inflammatory, fibrosclerosing neonatal cholangiopathy, characterized by a periductal infiltrate composed of CD4⁺ and CD8⁺ T cells. The pathogenesis of this disease has been proposed to involve a virus-induced, subsequent autoreactive T cell-mediated bile duct injury. Antigen-specific T-cell immunity involves clonal expansion of T cells expressing similar T-cell receptor (TCR) variable regions of the β -chain (V β). We hypothesized that the T cells in biliary atresia tissue expressed related TCRs, suggesting that the expansion was in direct response to antigenic stimulation.

Methods: The TCR V β repertoire of T cells from the liver, extrahepatic bile duct remnants, and peripheral blood of biliary atresia and other cholestatic disease controls were characterized by fluorescent-activated cell sorter analysis, and TCR junctional region nucleotide sequencing was performed on expanded TCR V β regions to confirm oligoclonality.

Results: FACS analysis revealed V β subset expansions of CD4⁺ and CD8⁺ T cells from the liver or bile duct remnant in all patients with biliary atresia and only 1 control. The CD4⁺ TCR expansions were limited to V β 3, -5, -9, and -12 T-cell subsets and the CD8⁺ TCR V β expansions were predominantly V β 20. Each V β subset expansion was composed of oligoclonal populations of T cells.

Conclusions: Biliary atresia is associated with oligoclonal expansions of CD4⁺ and CD8⁺ T cells within liver and extrahepatic bile duct remnant tissues, indicating the presence of activated T cells reacting to specific antigenic stimulation. Future studies entail identifying the specific antigen(s) responsible for T-cell activation and bile duct injury.

Biliary atresia (BA) is a progressive, inflammatory cholangiopathy of infancy that leads to fibrosis and obstruction of extra- and intrahepatic bile ducts with eventual biliary cirrhosis. Due to the progression of disease, it is the most common indication for liver transplantation in children. The etiology of BA is not known, but one theory regarding its pathogenesis is that bile duct damage occurs secondary to a viral-induced, autoreactive T-cell-mediated process directed against bile duct epithelia.¹⁻⁷ In the rotavirus-induced murine model of BA, we have recently demonstrated that adoptive transfer of hepatic T cells leads to a bile duct-specific inflammatory reaction in recipient severe combined immunodeficiency disease mice,

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suggesting cellular autoimmunity.⁸ To date, little information exists regarding the characterization of the T-cell populations infiltrating the intrahepatic and extrahepatic bile ducts in human BA and forms the basis of this study.

T cells express the T-cell receptor (TCR) for antigen on their surface, which is composed of a heterodimer of disulfide-linked α - and β -chains.⁹ Functional TCR α -chain (*TCRA*) and β -chain (*TCRB*) genes result from somatic rearrangement of germline gene segments during T-cell development. For example, *TCRB* genes are generated from the rearrangement of the variable (*BV*), diversity (*BD*), and junctional (*BJ*) region gene segments as well as random nucleotide additions and/or deletions at the junctions (Figure 1). This process makes the potential TCR repertoire enormous with greater than 10^7 sequence possibilities.¹⁰ Thus, it is highly improbable that any 2 T cells will express identical or similar TCRs, resulting in a heterogeneous TCR repertoire.^{11,12}

The highly variable *TCRB* junctional region forms the complementary determining region 3 (CDR3), which is critically involved in the TCR's interaction with the antigen/major histocompatibility complex (MHC). In response to the appropriate antigen/MHC combination, specific T cells become activated, are recruited to target organs, and undergo clonal expansion (ie, now many T cells express an identical TCR). These oligoclonal T-cell populations can often be identified through detection of an altered TCR $V\beta$ repertoire on organ-specific T cells.¹² Oligoclonal expansions have been identified in infection, autoimmunity, and malignancy. Increased expression of a particular TCR $V\beta$ family is a sign of clonal proliferation that can be confirmed through *TCRB* junctional region sequencing of the T-cell population.⁹⁻¹² In the present study, we hypothesized that T cells present in the portal tracts and extrahepatic bile duct remnant of BA patients are composed of oligoclonally expanded T-cell populations, suggesting their accumulation in response to conventional antigenic stimulation.

Materials and Methods

Study Population

Between 2004 and 2006, peripheral blood mononuclear cells (PBMCs), fresh liver wedge tissue, and extrahepatic bile duct remnant tissue were collected from 5 patients with BA at the time of the Kasai portoenterostomy. Extrahepatic bile duct remnant tissue only was obtained from 1 BA patient at the time of liver transplantation (BA 6). Liver wedge tissue was collected from 3 control patients with other cholestatic liver diseases, 2 subjects with TPN-related cholestasis and 1 with Alagille's syndrome. Extrahepatic bile duct tissue was collected from 3 control patients with bile duct lesions, 2 subjects with choledochal cysts at the time of surgical repair, and 1 with cystic fibrosis liver disease at time of liver transplant (Table 1). Exclusion criteria included known bacterial or viral infection at the time of surgery. This study was approved by the Colorado Multiple Institutional Review Board, The Children's Hospital, and informed consent was obtained from the parents.

Isolation and Culture of PBMC and Mononuclear Cells from Tissue

PBMCs were isolated by density gradient separation (Amersham, Uppsala, Sweden) and resuspended in a fluorescence-activated cell sorter (FACS) buffer for analysis. Liver and extrahepatic bile duct tissue were finely minced, and each individual piece was placed into a well of a 48-well plate with RPMI 1640 media (Invitrogen, Carlsbad, CA) supplemented with 20 U/mL of r-interleukin-2 (IL-2) (R&D Systems, Minneapolis, MN). T cells activated through TCR engagement become more responsive to IL-2, leading to their preferential expansion in culture.¹³ Tissue remained in culture for 2 weeks and 2×10^6 cultured immune cells were resuspended in FACS buffer while any remaining cells were cryopreserved for future T-cell subset isolation.

Immunofluorescence Analysis of TCR Variable Regions of the β -Chain ($V\beta$) Expression

PBMCs and cultured liver and duct remnant T cells were analyzed by 3-color immunofluorescence using monoclonal antibodies (mAbs) specific for CD4, CD8 (BD Biosciences, San Jose, CA), and TCR V region expression using mAbs directed against 16 different TCR $V\beta$ receptor subfamilies:¹⁴ $V\beta 2$ (MPB2D5), $V\beta 5.1$ (IMMU157), $V\beta 12$ (VER2.32.1), $V\beta 13.1$ (IMMU 222), $V\beta 14$ (CAS1.1.3), $V\beta 17$ (E17.5), $V\beta 20$ (ELL1.4), $V\beta 22$ (Immu546), Coulter-Immunotech, Fullerton, CA; $V\beta 3.1$ (8F10), $V\beta 5.2$ (F3.15.13), $V\beta 6.7$ (OT145), $V\beta 8.1$ (MX-6) (Endogen, Rockford, IL); $V\beta 7$ (3G5.5), $V\beta 13.2$ (H132.8), gifts from P. Marrack, Denver, CO; $V\beta 9$ (FIN9), $V\beta 23$ (AHU17), Pharmingen, San Jose, CA. The lymphocyte population was analyzed using a FACSCalibur cytometer (BD Biosciences, San Jose, CA) as previously described.¹² Tissue-specific TCR $V\beta$ expansions were defined as a ≥ 3 -fold increase in the level of expression of a particular $V\beta$ compared to its expression in blood.¹⁴

Analysis of TCRB Gene Expression in Tissue T Cells

Highly purified T-cell subsets ($CD3^+/CD4^+$, $CD3^+/CD8^+$) from previously cultured immune cells were obtained using a MoFlo FACS (Dako Cytomation, Carpinteria, CA) with a purity yield of 95%–99% of gated cells. Cellular RNA was extracted using Trizol as previously described⁶ and cDNA synthesized using the Advantage RT for polymerase chain reaction (PCR) kit (Clontech, Mountain View, CA). PCR products were generated utilizing HotStarTaq-polymerase (Qiagen, Valencia, CA), 3' primer CB (5'-TTCTGATGGCTCAAACAC-3') and the specified $V\beta$ 5' primer (see Supplemental Table 1 for primer sequences [see supplemental material online at www.gastrojournal.org]). Each PCR product was ligated into a pCR 2.1 cloning vector and transformed into competent *Escherichia coli* (TA Cloning Kit, Invitrogen, Carlsbad, CA). Twenty-four to 48 colonies containing insert were randomly selected for nucleotide sequencing. Cycle sequencing was performed using M13 reverse (5' -CAGGAAACAGCTATGAC-3') sequencing primer, dRhodamine sequencing solution, and an automated ABI 377 sequencer (Applied Biosystems Inc, Foster City, CA).

HLA Genotyping

High-resolution HLA genotyping (HLA-A, -B, -C, and HLA-DR, -DQ) was performed by standard molecular techniques at ClinImmune Labs (Aurora, CO).

Statistical Analysis

All quantification of T-cell subsets is shown as the mean $\pm$ SEM. The following statistical methods were utilized to determine the significance of differences between the various study groups: Mann–Whitney test for cell surface marker expression, Student's *t* test for total T-cell populations and Fisher exact test for the presence or absence of TCR $V\beta$ expansions (In Stat Software, San Diego, CA). A *P* value of $< .05$ was considered statistically significant.

Results

Subtype and Distribution of Tissue T Cells in BA

Previous studies of BA have shown that both the extrahepatic and intrahepatic bile ducts are surrounded by a mixed inflammatory cellular infiltrate, composed primarily of $CD4^+$ and $CD8^+$ T cells and macrophages.^{6,15-17} In this study, the presence of periductal inflammation was documented by H&E staining of a portion of each tissue specimen collected, as shown in Figure 2. Extensive periductal inflammation was observed within the portal tracts and extrahepatic bile duct remnants of BA patients. Liver tissue controls likewise had portal tract

infiltrates; however, minimal periductal inflammation was observed in the extrahepatic bile duct controls.

In BA, the infiltrating portal tract T cells are highly activated¹⁵; however, the relative distribution and phenotype of these activated T cells was not known. Therefore, we next determined the yield and subtype (CD4⁺ or CD8⁺) of activated T cells in BA and control specimens. The liver and extrahepatic bile duct remnant tissues were placed in culture for 2 weeks in the presence of IL-2, leading to the preferential expansion of previously activated T cells. The amount of BA or control liver tissue placed in culture was approximately 10–20 mg for each specimen. Greater numbers of immune cells were isolated from BA liver tissue (mean $\pm$ SEM: $5.1 \pm 1.9 \times 10^6$) compared with controls ($3.0 \pm 0.6 \times 10^6$) (Figure 3A). Approximately 20 mg of extrahepatic bile duct remnant tissue from BA patients was cultured compared to 200 mg of extrahepatic bile duct tissue from controls. Despite this 10-fold greater amount of tissue available from the controls, the BA duct tissue yielded almost twice as many immune cells in culture (BA: $3.6 \pm 0.7 \times 10^6$; controls: $1.9 \pm 0.6 \times 10^6$).

Analysis of the types of immune cells cultured from the tissue revealed that the majority of the cells were CD3⁺ T cells. BA livers contained significantly more CD3⁺ T cells compared to control livers (CD3⁺: BA, $79.5 \pm 3.5\%$; controls, $53.9 \pm 3.6\%$, $P = .003$). However, similar percentages of T cells were found in the BA bile duct remnant and controls (Figure 3B). The normal liver also harbors a generous amount of natural killer (NK) cells, and thus, further analysis of the cultured liver cells for NK cells and NKT cells was performed. Significantly less NK cells (CD56⁺CD3[−]) were present in BA livers compared to controls (BA, $8.8 \pm 3.9\%$; controls, $32 \pm 4.8\%$, $P = .007$). Regarding NKT cells, the majority of liver NKT cells have a restricted TCR- α chain expressing V α 24 and this cell surface marker is used routinely to identify classic NKT cells.¹⁸ No expansions of TCR V α 24⁺ cells within cultured lymphocytes were identified in either group (BA, $0.50 \pm 0.14\%$; controls, $0.47 \pm 0.12\%$).

Quantification of T-cell subtypes was determined by FACS analysis, and the percentage of CD3⁺ T cells expressing CD4 or CD8 was determined. Representative FACS density plots of cultured cells from BA and controls are shown in Figure 3C. Significantly less CD4⁺ T cells ($24.4 \pm 3.1\%$) and more CD8⁺ T cells ($38.1 \pm 5.6\%$) were present from BA liver tissue compared to controls (CD4: $60.4 \pm 8.1\%$; CD8: $17.9 \pm 1.9\%$) ($P < .05$). In contrast, cells from the BA extrahepatic bile duct remnants were mainly composed of CD4⁺ T cells ($50.9 \pm 7.9\%$), the predominant phenotype also present in control duct tissues ($55.8 \pm 10.2\%$) (Figure 3D).

Limited TCR V β Repertoire Expression on CD4⁺ T Cells from BA Livers and Extrahepatic Bile Duct Remnants

To identify which TCR V β subsets may harbor populations of oligoclonally expanded T cells, PBMCs and cultured liver and bile duct remnant T cells were analyzed for CD4 and TCR V β region expression by FACS analysis using mAbs directed against 16 different TCR V β subsets. For all studies, TCR V β expression of organ-specific T cells was considered significantly increased when the percentage of a specific TCR V β was greater than 3-fold over baseline TCR V β expression from the patient's PBMCs.^{12,14} As a control, to determine if culturing cells in the presence of IL-2 would skew TCR V β cell surface expression, perihepatic lymph nodes isolated from 2 patients were cultured for 2 weeks in media alone or media supplemented with IL-2, followed by TCR V β analysis of both CD4⁺ and CD8⁺ T cells. No skewing of the TCR V β repertoire was observed in lymph node T cells cultured for 2 weeks in the presence of IL-2 compared with media alone or PBMCs (data not shown).

TCR V β repertoire analysis indicated that all BA tissue specimens (livers or bile duct remnants) contained 1 to 2 significant CD4⁺ TCR V β expansions (Figure 4A). The predominant V β subsets expanded included V β s 3, 5, 9, and 12. Patient BA6 had the greatest expansion of a

single $V\beta$, with over 50% of all $CD4^+$ T cells from the bile duct remnant expressing $V\beta 12$, compared with 2% of the PBMCs.

In contrast to BA, only 1 of the 6 control tissues demonstrated a $CD4^+$ TCR $V\beta$ expansion. Figure 4B shows the percentage of $CD4^+$ T cells expressing each of the $V\beta$ subsets from control tissues. A single choledochal cyst remnant had a significant expansion of $V\beta 17$ -expressing $CD4^+$ T cells, with 26% of these cells from the bile duct expressing $V\beta 17$ compared with 5.1% of PBMCs. To summarize, all 6 BA patients demonstrated at least 1 $CD4^+$ T cell expansion compared with only 1 expansion in the 6 control patients ($P = .015$). The $CD4^+$ T cells found within diseased liver and bile duct remnants of BA subjects utilized a limited TCR $V\beta$ repertoire, suggesting that the distribution of $V\beta$ s found was not random but in response to an antigen driven expansion.

TCR CDR3 Sequence Analysis Confirms Oligoclonality of Expanded $CD4^+$ TCR $V\beta$ Subsets in BA

To determine if the TCR $V\beta$ expansions identified in BA contained oligoclonal populations, the *TCRB* junctional regions were sequenced. The prevalent, deduced amino acid sequences of the different clones are summarized in Figure 5, with the complete sequences of all identified clones in Supplemental Figure 1 (see supplemental material online at www.gastrojournal.org). All TCR $V\beta$ expansions identified by FACS analysis were confirmed to be oligoclonal by nucleotide sequence determination. A minimum of 3 identical sequences from different clones defined a clonal expansion.^{12,14} Dominant clones that constituted $\geq 40\%$ of a particular $CD4^+$ TCR $V\beta$ subset were present in 11 of 13 (84%) BA samples tested. In several instances, almost the entire $V\beta$ subset was dominated by a single clone with $\geq 90\%$ of clones expressing identical TCRs (specimens BA1, BA3, and BA6).

Striking Expansions of $CD8^+$ T Cells Expressing TCR $V\beta 20$ in BA Liver and Extrahepatic Bile Duct Remnants

TCR $V\beta$ subsets expressed on $CD8^+$ T cells were also analyzed by FACS analysis to determine the presence of $V\beta$ expansions in this T cell subset. As shown in Figure 6A, 4 of the 6 BA specimens revealed striking expansions of $CD8^+$ T cells expressing $V\beta 20$ from the liver and/or bile duct remnant, compared to autologous PBMCs. In specimens BA1, BA3, and BA5, both the liver and bile duct remnant contained $CD8^+$ TCR $V\beta 20$ expansions, while it was present only in the liver in BA 2. Furthermore, although not classified as an expansion by our definition, a 2.2-fold increase in TCR $V\beta 20$ expression on $CD8^+$ T cells from the extrahepatic bile duct remnant tissue of patient BA6 was also observed. In contrast, control livers and extrahepatic bile ducts did not contain $CD8^+$ TCR $V\beta$ expansions compared to autologous PBMCs (Figure 6B).

TCR CDR3 Sequence Analysis Confirms Oligoclonality of the Expanded $CD8^+$ TCR $V\beta 20$ in BA

Oligoclonality of the $CD8^+$ TCR $V\beta 20$ expansions found in BA samples was confirmed by sequence analysis of the *TCRB* junctional regions (Figure 7). $CD8^+$ TCR $V\beta 20$ expansions were verified to be oligoclonal in nature by demonstrating at least 3 identical sequences from different clones. Dominant clones that constituted $\geq 40\%$ of a specific $CD8^+$ TCR $V\beta$ were present in 4 of 8 (50%) BA samples tested, with a frequency of identical clones ranging from 13%–81% within a specimen.

Taken together, our findings demonstrate that the lymphocytes in the livers and extrahepatic bile duct remnants of BA patients include a unique subset of previously activated oligoclonal $CD4^+$ and $CD8^+$ T cells, signifying expansion in response to conventional antigenic stimulation.

HLA Genotypes of Patients with V β Subset Expansions

The particular TCR variable region expressed by expanded T cells is a consequence of either the stimulating antigen and/or the MHC-presenting molecule. It was possible, therefore, that the predominant CD8⁺ TCR V β 20 expression or the limited CD4⁺ TCR V β repertoire present in BA patients was related to a common HLA genotype. Table 2 summarizes the biliary atresia CD8⁺ and CD4⁺ TCR V β expansions and compares them with the MHC class I and II genotypes of these patients. No obvious association between MHC class I genotype and CD8⁺ TCR V β 20 was observed. Interestingly, for MHC class II genotypes, 2 associations were observed. Patients BA1 and BA6 both had significant expansions of CD4⁺ T cells expressing V β 12 and shared the *HLA-DRB1**0701/*DQB1**0202 alleles. Patients BA3 and BA4 both had significant expansion of TCR V β 3 and shared *HLADQB1**0301. However, the limited number of specimens available for HLA analysis in this study group prohibits any conclusion regarding a definite association of HLA genotypes and BA.

Discussion

We have postulated that the pathogenesis of BA involves a viral-induced, progressive autoimmune-mediated injury of bile duct epithelia.¹ In this scenario, a perinatal infection with a virus that is tropic for the bile duct epithelia would cause initial bile duct epithelial injury. Despite viral clearance, persistent inflammation and injury to the bile duct epithelia would ensue. The damaged bile duct epithelial cells may express either altered or previously sequestered “self”-antigens that are subsequently recognized as foreign, eliciting autoreactive T-cell-mediated inflammation (bystander activation pathway of autoimmunity). Alternatively, viral proteins may be structurally similar to bile duct epithelial proteins and autoimmunity could be elicited based on the molecular mimicry pathway. In support of this autoimmune theory, we have recently shown evidence for both cellular and humoral autoimmunity in a rotavirus-induced murine model of BA.⁸

Oligoclonal T-cell expansions are found in antigen-driven immune responses, including autoimmune diseases, infections, and malignancies.¹¹ In this context, our aim was to determine if the organ-specific T cells in BA were oligoclonal in nature, lending support to the pathogenesis theory described above. We found that the activated T cells from BA tissue were composed of both CD4⁺ and CD8⁺ T cell subsets within the liver and predominantly CD4⁺ T cells within extrahepatic bile duct remnants. All BA patients demonstrated CD4⁺ T-cell expansions and 5 of the 6 patients had CD8⁺ T cell expansions. Both T-cell subsets were found to be oligoclonal in nature with the CD4⁺ T cells displaying a limited TCR V β repertoire and the expanded CD8⁺ T cells expressing mainly TCR V β 20. Given the enormous diversity of the human TCR repertoire, the identification of oligoclonal T-cell populations in BA strongly suggests that the T cells are being actively recruited to the liver and bile duct in response to conventional antigen recognition. In addition, the limited number of V β -expressing subsets in these BA patients suggests that the dominant immune response is directed against a finite number of antigens.

The nature of the antigen(s) being recognized by the oligoclonal T cells in BA is not known. Many researchers have demonstrated oligoclonality with a limited TCR V β repertoire in CD4⁺ T-cell populations from CD4⁺ T-cell-driven autoimmune diseases including autoimmune hepatitis,^{19,20} Crohn's disease,²¹ and multiple sclerosis.²²⁻²⁴ Interestingly, in our study 3 of the 5 BA patients had different CD4 TCR V β subset expansions within their liver compared with their own extrahepatic bile duct remnant. The different usage of V β subsets in the context of the same MHC may reflect different peptides being recognized at the site of pathology. This would not be unexpected, as many autoimmune diseases such as multiple sclerosis and type 1 diabetes mellitus have more than 1 potential stimulating autoantigen.¹¹ Furthermore, concurrent CD8⁺ T-cell oligoclonality has been shown for multiple

sclerosis^{25,26} and other autoimmune processes including aplastic anemia²⁷ and juvenile dermatomyositis syndrome.²⁸ The predominance of CD8⁺ TCR V β 20 in our BA population also points to a possible role of a viral antigen activating the CD8⁺ T cells. Future studies aimed at identifying the inciting antigen(s) responsible for T-cell activation will entail analyzing candidate viral antigens including reovirus,^{3,4,29} rotavirus,⁵ and cytomegalovirus,³⁰ as well as bile duct epithelial autoantigens.

Potential limitations to this study include both the inability to determine the ex vivo TCR V β repertoire of target organ T cells due to the small size of tissue samples and the methods used to enhance lymphocyte growth from tissue specimens. Antigen-independent IL-2 stimulated in vitro expansion of T cells leads to the preferential release and growth of activated T cells from diseased tissue. In other diseases characterized by the absence of a known antigenic stimulus such as rheumatoid arthritis³¹ or autoimmune hepatitis,¹⁹ IL-2-induced T-cell expansion has been used to study the TCR repertoire. Several observations support the validity of using this experimental technique to expand disease-relevant T cells in BA. First, elegant studies by Arenz et al³² have shown that antigen-independent IL-2 stimulation does not alter the TCR repertoire. In those studies, analysis of TCR V β subsets on PBMCs activated in culture with PHA, anti-CD3, or IL-2 failed to skew the V β repertoire from un-stimulated controls. Second, in our own studies, no significant differences in the TCR repertoire were identified from lymph node cells cultured in the absence or presence of IL-2. Finally, if culturing of T cells in the presence of IL-2 were to result in nonspecific TCR V β expansions, one would have expected to see expansions in all of the control tissues, which was not observed in this study.

Another potential limitation to this study is that we were not able to analyze all of the TCR V β subfamilies, and theoretically may have failed to identify an important T-cell expansion with the TCR V β antibodies used. The human TCRB repertoire consists of approximately 47 functional V gene segments (V β s) distributed in a total of 21–23 subfamilies.³³ Many of these gene segments are infrequently expressed. The 16 different TCR V β subfamilies analyzed in our study cover about 2/3 of the expressed TCR repertoire. Despite this theoretic limitation, we identified at least 1 expansion in all BA patients. Furthermore, if our analysis had missed large expansions of TCR V β subfamilies not covered by our panel of antibodies, then one would expect a reciprocal decrease in all other V β subfamilies within the tissue compared with PBMCs. However, this phenomenon was not observed in BA or control tissues.

In summary, BA is associated with oligoclonal expansions of CD4⁺ and CD8⁺ T cells within liver and extra-hepatic bile duct remnant tissues, indicating the presence of activated T cells reacting to specific antigenic stimulation. Identifying the specific antigen(s) responsible for T-cell activation will form the basis of future studies and lead to an enriched understanding of the role of T cells in the bile duct injury prevalent in this disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1053/j.gastro.2007.04.032.

Abbreviations used in this paper

BA, biliary atresia; FACS, fluorescent-activated cell sorter; IL-2, interleukin-2; mAbs, monoclonal antibodies; MHC, major histocompatibility complex; NK, natural killer; PBMCs, peripheral blood mononuclear cells; PCR, polymerase chain reaction; TCR, T-cell receptor; V β , variable region of the β -chain.

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Figure 1.

The sequence of events in recombination and gene expression of the TCR β -chain. Expressed TCR β -chain (TCRB) genes are generated from the rearrangement of variable (V) to diversity (D) to junctional (J) region gene segments. Further diversification is generated from the presence of random nucleotide additions and deletions at the V-to-D and D-to-J joining points. Shown here is an example of the *TCRB* region encoded by the selection of exons V β 1, D β 1, the second exon in the J β 1 cluster, and the constant region C β 1. This process makes the potential TCR repertoire enormous with greater than 10^7 sequence possibilities.

Figure 2.

Representative histology of liver and extrahepatic bile duct remnant from BA and control tissue. Marked inflammation was observed within the portal tracts (A) and extra-hepatic bile duct remnant (B) of BA patients. Intense portal tract inflammation was also seen within the liver of control subjects (C, TPN-related cholestasis liver), while mild inflammation was present in extrahepatic bile duct tissue from control subjects (D, choledochal cyst).

Figure 3.

High yield of immune cells from BA tissue with a predominance of CD8⁺ T cells in liver tissue and CD4⁺ T cells in the extrahepatic duct remnant. (A) After 2 weeks in culture with IL-2, total cell yield was determined with a hemocytometer. Greater numbers of immune cells were obtained from BA livers and extrahepatic duct remnants compared to controls. (B) Percentage of total cells isolated in culture that were positive for the T-cell marker CD3. Significantly more CD3⁺ T cells were present in the liver from BA patients compared to controls (**P* < .05). (C) Representative FACS analysis density plots of isolated T cells from BA specimens and controls, gated on all cells. Shown is a representative percentage of CD3⁺ T cells that were CD4 or CD8 positive in each group. (D) Summary of cell surface expression of CD3, CD4,

and CD8. Significantly greater percentages of CD3⁺CD8⁺ T cells and smaller percentages of CD3⁺CD4⁺ T cells were detected from BA livers compared with liver controls (**P* < .05).

Figure 4.

Expansion of CD4⁺ T cells with limited V β repertoire in BA livers and extrahepatic duct remnants. TCR V β repertoire of cultured CD4⁺ T cells from liver (*shaded black*), bile duct tissue (*shaded gray*), and PBMCs (*white*) in BA patients (*A*) and controls (*B*). Asterisks denote expanded TCR V β regions (>3-fold increase over PBMCs) in individual patients. All BA tissue contained 1 or 2 CD4⁺ TCR V β expansions. Shown in *B* are the mean $\pm$ SEM percent of CD4⁺ T cells expressing each of the V β subtypes from control tissues. Only 1 control tissue (choledochal cyst bile duct) had a significant CD4⁺ TCR V β expansion (V β 17).

Deduced amino acid sequences

Patient/tissue	TCRBV	BV	nBDn	BJ	TCRBJ	#/total	%freq.
BA1/liver	9	CASS	QAGGN	GYTFG	1.2	6/22	27%
		CASS	QVIGG	SYEQYFG	2.7	6/22	27%
BA1/duct	9	CASS	LAGG	STDTQYFG	2.3	18/21	95%
	12	CAIS	EGA	NTGELFFG	2.2	23/32	84%
BA2/liver	6	CASS	PPKWLGA	EAFFG	1.1	12/27	44%
	7	CASS	QGID	TEAFFG	1.1	19/30	63%
BA3/liver	9	CASS	RTSRG	SGNTIYFG	1.3	36/40	90%
BA3/duct	3	CASS	PWTSGSF	TDTQFG	2.3	39/41	95%
BA4/liver	3	CASS	QRIP	YEQYFG	2.7	10/25	40%
	17	CASS	LSSR	SYNSPLHFG	1.6	22/29	76%
BA4/duct	5	CASS	SLRDRG	NIQYFG	2.4	18/22	82%
BA5/liver	5	CASS	SHDREG	TQTFG	2.5	18/28	64%
BA5/duct	5	CASS	LNS	NSPLHFG	1.6	4/36	11%
		CASS	PTGWA	YEQYFG	1.6	4/36	11%
BA6/duct	12	CAI	TSG	NTGELFFG	2.2	44/45	98%

Figure 5.

TCRB junctional region amino acid sequences expressed in CD4⁺ T cells from liver and extrahepatic duct remnants of BA patients. Dominant *TCRB* sequences are shown here. For each T-cell clone the entire *TCRB* junctional region is shown, extending from the 5' end of the selected *TCRBV* family gene, including the highly rearranged *nBDn* gene segment, and ending at the selected *BJ* gene segment. The column *TCRBJ* denotes for each clone for which a *BJ* gene family member was selected during genetic rearrangement. The number of identical sequences is shown over the total number of sequences analyzed for a given sample and is depicted as percentage of frequency.

Figure 6.

Striking expansion of CD8⁺ TCR V β 20 in BA liver and extra-hepatic duct remnants. TCR V β repertoire of cultured CD8⁺ T cells from livers (*shaded black*) and bile duct tissue (*shaded gray*) compared with autologous PBMCs (*white*) in BA patients (*A*) and controls (*B*). Asterisks denote the expanded TCR V β region (>3-fold increase over PBMCs) in individual patients. Four of 6 BA specimens revealed striking expansions of CD8⁺ TCR V β 20 within the liver and ductal remnants compared with autologous PBMCs. Shown in *B* are the mean $\pm$ SEM percent of CD8⁺ T cells expressing each of the V β subsets from control tissues. None of the controls contained CD8⁺ TCR V β expansions.

Deduced amino acid sequences							
Patient/tissue	TCRBV	BV	nBDn	BJ	TCRBJ	#/total	%freq.
BA1/liver	20	CAW	ESGAD	EQYFG	2.7	12/38	32%
BA1/duct	20	CAWS	GLAGG	NTGELFFG	2.2	16/39	41%
BA2/liver	20	CAW	DRGSVM	NTEAFFG	1.1	30/37	81%
BA3/liver	20	CA	SWRD	SYNEQFFG	2.1	5/30	17%
		CAWS	TPRLAGGPD	NEQFFG	2.1	4/30	13%
BA3/duct	20	CAWS	VDGG	QPQHFG	1.5	7/31	23%
		CAW	TSLAKSV	EQFFG	2.1	5/31	16%
		CAWS	ERA	YNEQFFG	2.1	5/31	16%
BA5/duct	20	CAWS	LGASVSA	YNEQFFG	2.1	14/20	70%
BA6/duct	20	CAS	G	EQFFG	2.1	13/18	72%
BA2/liver	13	CAS	AARPAS	YGYTFG	1.2	32/36	89%

Figure 7.

TCRB junctional region amino acid sequences expressed in CD8⁺ T cells expressing TCR V β 20 from liver and extrahepatic duct remnants of BA patients. CD8⁺ TCR V β 20 *TCRB* sequences found 3 times or more in any sample are shown here. For each T-cell clone the entire *TCRB* junctional region is shown. The column *TCRBJ* denotes for each clone for which a *BJ* gene family member was selected during genetic rearrangement. The number of identical sequences is shown over the total number of sequences analyzed for a given sample and is depicted as percentage frequency.

Table 1

Characteristics of Study Subjects

Patient	Age	Diagnosis	Bilirubin (mg/dL-tot./direct)
BA1	9.5 wk	Biliary atresia	11.5/8.3
BA2	10 wk	Biliary atresia	7.9/6.2
BA3	6 wk	Biliary atresia	8.3/5.9
BA4	9.5 wk	Biliary atresia	7.1/5.2
BA5	16 wk	Biliary atresia	11.1/6.4
BA6	11 mon	Biliary atresia	16.3/11.8
Control liver 1	10 wk	TPN-related cholestasis	14.9/12.3
Control liver 2	4 wk	TPN-related cholestasis	4.8/4
Control liver 3	24 mon	Alagille's syndrome	14.3/11.7
Control duct 1	3 yr	Choledochal cyst	1/0.2
Control duct 2	16 yr	Choledochal cyst	0.4/0.1
Control duct 3	12 mon	Cystic fibrosis	57.3/36

Table 2

HLA Genotyping of Biliary Atresia Patients

Patient	Dominant CD8 ⁺ Vβ	HLA-A	HLA-B	HLA-Cw
BA1	20	0301, 1101	0801, 3501	0401, 0701
BA2	13, 2, 20	2402, 3101	3901, 5102	0801, 1203
BA3	20	0201, 0301	1501, 3502	0303, 0602
BA4	—	0101, 0201	3501, 5701	0401, 0602
BA5	20	0101, 0201	0801, 5201	0303, 0701
BA6	7, 20	0201, 3101	1515, 5101	0102, 1402
Patient	Dominant CD4 ⁺ Vβ	HLA-DRB1		HLA-DQB1
BA1	9, 12	0701, 1301	0701, 1301	0202, 0603
BA2	6, 7	1201, 1402	1201, 1402	0301
BA3	3, 9	0103, 1104	0103, 1104	0301, 0501
BA4	3, 5, 17	0701, 1101	0701, 1101	0301, 0303
BA5	5	0301, 1401	0301, 1401	0201, 0503
BA6	12	0701, 0802	0701, 0802	0202, 0402